Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/020152

International filing date: 23 June 2004 (23.06.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/480,406
Filing date: 23 June 2003 (23.06.2003)

Date of receipt at the International Bureau: 30 August 2004 (30.08.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1214151

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

August 19, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/480,406*
FILING DATE: *June 23, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/20152*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

Mail Stop Provisional Patent Application

PTO/SB/16 (6-95)
Approved for use through 04/11/98. OMB 0651-0037
Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

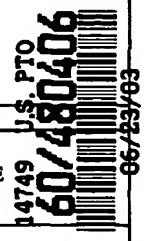
PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (c).

	Docket Number	2805-4	Type a plus sign (+) inside this box→	+

INVENTOR(S)/APPLICANT(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
CHRISTENSEN	Tim	W.	Ithacia, New York



TITLE OF THE INVENTION (280 characters)

METHODS FOR OBTAINING WHOLE-ORGANISM THERAPEUTIC AGENTS USING NEAR OR SUPERCRITICAL CARBON DIOXIDE

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☒ **Customer Number:** **23117**

Type Customer Number here

Place Customer Number Bar Label Here →

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒	Specification	Number of Pages	18	☐	Applicant claims "small entity" status. ☐ "Small entity" statement attached.
☒	Drawing(s)	Number of Sheets	3	☒	Other (specify) Application Title Page

METHOD OF PAYMENT *(check one)*

☒	A check or money order is enclosed to cover the Provisional filing fees ($160.00)/($80.00)	PROVISIONAL FILING FEE AMOUNT ($) 160.00
☐	The commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 14-1140	

The invention was made by an agency of the United States Government or under a contact with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE _____ DATE June 23, 2003

TYPED or PRINTED NAME Bryan H. Davidson

REGISTRATION NO. *(if appropriate)* 30,251

☐ Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

Burden Hour Statement: This form is estimated to take .2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Mail Stop Comments - Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, and to the Office of Information and Regulatory Affairs, Office of Management and Budget (Project 0651-0037), Washington, DC 20503. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: **Mail Stop Provisional Patent Application**, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Atty. Dkt. No.: 2805-4

U.S. PROVISIONAL PATENT APPLICATION

Inventor: **Tim W. CHRISTENSEN**

Invention: **METHODS FOR OBTAINING WHOLE-ORGANISM THERAPEUTIC AGENTS USING NEAR OR SUPERCRITICAL CARBON DIOXIDE**

NIXON & VANDERHYE P.C.
ATTORNEYS AT LAW
1100 NORTH GLEBE ROAD
8TH FLOOR
ARLINGTON, VIRGINIA 22201-4714
(703) 816-4000
Telecopier (703) 816-4100

SPECIFICATION

METHODS FOR OBTAINING WHOLE-ORGANISM THERAPEUTIC AGENTS USING NEAR OR SUPER CRITICAL CARBON DIOXIDE

FIELD OF THE INVENTION

The present invention relates generally to methods whereby whole-organism therapeutic agents may be obtained by use of near or super critical carbon dioxide.

BACKGROUND AND SUMMARY OF THE INVENTION[1]

Vaccines represent one of the seminal developments in humankind's ongoing battle against disease. Vaccination is still the best defense against existing, novel, and manipulated pathogens. The earliest whole-cell vaccines were prepared by inactivating a given pathogen using heat or chemical treatments. Whole-cell vaccines have significant advantages over attenuated and subunit vaccines. Thermal or chemical inactivation of the pathogen is simple and inexpensive, and provides rapid access to a vaccine. Both subunit vaccines and vaccines based on attenuated strains require considerable time and expense before they can be put to use. Despite the advantages of chemical inactivation, chemically inactivated vaccines sometimes fail to elicit robust and protective immune responses [2-4]. The addition of adjuvants to these preparations may boost the immune response, but immunity is still insufficient in many cases and may require frequent boosting.

Many complications associated with chemically inactivated vaccines arise from the simple fact that inactivation alters the chemical

[1] The entirety of each publication cited below is expressly incorporated hereinto by reference.

properties of key antigens required to elicit a protective immune response. The development of a rapid, inexpensive, and effective method to inactivate a pathogen while maintaining the integrity of its surface antigens would represent a powerful new tool in vaccine development.

Recent work has effectively demonstrated that microbes inactivated by a non-denaturing method do, in fact, elicit more robust immune responses than chemically inactivated pathogens [5]. "Ghosts," as they are known colloquially, are the empty shells of microbes that have been inactivated by the controlled expression of the PhiX174 lysis gene "E" [6]. Essentially the cytoplasmic contents of the cells are expelled via the transmembrane tunnel formed by the lysis protein [6]. Vaccines prepared through this genetic manipulation have been shown to be superior to chemically inactivated pathogens, most likely due to the non-denaturing inactivation procedure [3]. Moreover, it is hypothesized that the more robust immune response is not simply a function of individual proteins, but also is related to the method of presentation.

Cell walls remain largely intact, native surface antigens are preserved, and bioadhesive properties are likely maintained in ghost vaccine preparations. All of these characteristics endow ghost vaccines with inherent adjuvant properties that contribute to protective immune responses [3, 7-17]. The usefulness of the bacterial ghost system is extended by inactivating bacteria expressing antigens that are derived from other pathogens. The end result is a vaccine with inherent adjuvant properties that is protective against any number of desired bacterial, viral, protozoan, and fungal pathogens [12, 13, 15, 16, 18]. There are concerns about the endotoxicity of Lipid A and Lipopolysaccharide (LPS) in these whole cell vaccines. However, it has been demonstrated that endotoxicity is not a real limit to the use of ghost vaccines [17].

Despite it's promise, the ghost vaccine technology exhibits a number of drawbacks. The first of these concerns centers on safety. The lysis system employed typically results in only a 4 log reduction in colony forming units (CFUs) [5]. The remaining organisms must be inactivated by further processing. This may or may not be the case. The ghost system uses an additional kill mechanism to inactivate the remaining survivors [5]. This layering of genetic systems in the ghost technology is a cause for additional concern. Because these genetic systems are maintained within the chosen cells by selection on various antibiotic containing media [19, 20], lateral transfer of antibiotic resistance to other pathogens within an individual is a possibility [21].

In addition to safety concerns, the ghost system only works with Gram-negative bacteria. Furthermore, genetic manipulation of additional serotypes may be required to generate a broadly protective vaccine. Therefore, the applicability of the ghost technology is limited to the gram-negative bacteria that are tractable to genetic manipulation. These limitations preclude a significant number of pathogens, notably: *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Enterococcus sp., Bacillus anthracis, Bacillus cereus, Lactobacillus sp., Listeria monocytogenes, Nocardia sp., Rhodococcus equi, Erysipelothrix rhusiopathiae, Corynebacterium diptheriae, Propionibacterium acnes, Actinomyces sp. Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani*, and *Peptostreptococcus sp.* The applicability of ghost vaccine technologies is further limited by the method's failure to inactivate spores, which are insensitive to induction of lysis genes due to their dormant nature.

Whole-cell vaccines based on the ghost methodology are superior to chemically inactivated pathogens, but they cannot be produced rapidly. Even if the microbe is previously known, considerable time and expense are required to generate a new ghost vaccine for a given pathogen, especially for novel or genetically intractable pathogens.

The need for new and broadly applicable inactivation technologies is exacerbated by the very nature of biological weapons. The pathogens that are, or may be employed as bio-warfare and bio-terror agents such as Anthrax, Tularemia, Botulism, Plague, Epsilon toxin, Q fever, enterotoxin B, Typhus fever, Melioidosis, and Brucellosis are not usually endemic diseases in humans. As such there is very little, if any, commercial advantage to generating vaccines using expensive and time-consuming techniques. An appealing alternative that would speed the production of such vaccines and enable quick response to emerging serotypes is an inactivation technology that in and of itself generates high quality vaccines. Bacterial inactivation by supercritical CO2 may represent such a technology. The technology for using supercritical CO2 is well-known and has been adapted to large industrial applications, including the extraction of natural compounds from plant materials [22] and detoxification of contaminated soil [23]. Supercritical CO2 applications have also found their way into medical circles as a method for bone de-lipidation [24], drug manufacture [25], and sterilization among others [1]. The first attempts to use supercritical CO2 as a sterilant resulted in inadequate levels of inactivation [26].

Recently, in U.S. Patent No. 6,149,864 to Dillow et al (the entire content of which is expressly incorporated hereinto by reference), the use of supercritical CO_2 was disclosed as an alternative to existing technologies for sterilizing a wide range of products for the healthcare

industry with little or no adverse effects on the material treated. Specifically, the Dillow '864 patent disclosed the inactivation of a wide range of vegetative microbial cells using supercritical carbon dioxide with agitation and pressure cycling. However, only one spore-forming bacterium was investigated in the Dillow '864 patent, specifically, *B. cereus*. No disclosure appears in Dillow '864 patent regarding the efficacy of the therein suggested techniques using currently accepted bio-indicator standards used to judge sterilization (i.e., *B. stearothermophilus* and *B. subtilis*). Subsequently, however, other investigators achieved only a 3.5 log reduction in *B. subtilis* spores using the method disclosed in the Dillow et al '864 patent [27].

In addition to bacterial inactivation, viral inactivation is realized using supercritical CO2 [28]. Moreover it has been shown that sterilization by supercritical CO2 does not affect the properties of a biodegradable polymer (PLGA) and leaves bacterial cells intact [1].

It would therefore be desirable if methods could be provided whereby organisms are inactivated utilizing near or supercritical CO2 for the purpose of generating whole-cell therapeutic agents. It is towards fulfilling such a need that the present invention is directed.

In general, the methods of the present invention result in whole-organism therapeutic agents by treatment of the organisms using near or super critical carbon dioxide. In preferred embodiments, the methods of this invention treat organisims with near or supercritical CO2 at pressures between about 1000 to about 3500 psi, at temperatures in the range of between about 25^{0}C to 60^{0}C, and times ranging from 20 minutes to 12 hours. In especially preferred embodiments, the present invention utilizes the techniques disclosed in commonly owned U.S. Provisional Application

Serial No. 60/---,--- filed even date herewith (Atty. Dkt. No. 2805-3), the entire content of which is expressly incorporated hereinto by reference.

These and other aspects and advantages will become more apparent after careful consideration is given to the following detailed description of the preferred exemplary embodiments thereof.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Reference will hereinafter be made to the accompanying drawings, wherein like reference numerals throughout the various FIGURES denote like structural elements, and wherein;

FIGURE 1 is a schematic view of a presently preferred sterilization apparatus in accordance with the present invention;

FIGURE 2 is a detailed schematic view of the pressure vessel employed in the apparatus of FIGURE1;

FIGURES 3A-3F are scanning electron microscope (SEM) micrographs of *S. aureus, P. aeruginosa* and *E. coli* spores before and after inactivation with supercritical CO2 in accordance with Example 2 below; and

FIGURES 4A and 4B are respectively a SEM micrograph of inactivated *B. subtilis* spores and vegetative cells, and a comparison of total protein profiles of untreated vs. supercritical CO2 inactivated *B. subtilis* spores and vegetative cells (Comassie stained 12% SDS-page gel of total protein extracted) in accordance with Example 3 below.

DETAILED DESCRIPTION OF THE INVENTION

As noted previously, the present invention results in the inactivation of organisms for the purposes of generating whole-cell therapeutic agents. These organisms include bacteria, viruses, fungi, protozoa, and helminths. The method and apparatus provided utilizes treatment with near or super critical CO2.

A wide range of organisms can be inactivated using the methods of the present invention, including for example, gram-positive bacteria, gram-negative bacteria, fungi, protozoa, viruses, protozoa, and helminths. Given the low temperatures and low pressures, inactivation by supercritical CO2 using the method of the present invention is especially useful to generate whole-cell therapeutics while maintaining the properties of thermally labile and/or hydrolytically labile materials.

Given the existing literature and corollaries to the ghost technology for vaccine production it is possible to generate some predictions about the properties of supercritical CO2 inactivated microbes as vaccines (Table 1).

Table 1

Comparison of formalin, ghost, and supercritical CO2 inactivation technologies for key aspects in applicability to production of high quality vaccine preparations

	Method of Inactivation		
	Formalin	**Ghost**	**Supercritical CO2**
Speed of development	+	-	+
Non-denaturing	-	+	+
>6 Log reduction in CFUs	+	-	+
Inactivation: Gram negative bacteria	+	+/-	+
Gram positive bacteria	+	-	+
Viruses	+	-	+
Maintains structural properties of bacterium	-	+	+
Adjuvant properties	-	+	+
No chemical residues	-	+	+
Lacks antibiotic resistance genes	+	-	+

As noted previously, the present invention contemplates subjecting whole organisms or microbes to pressure and temperature conditions in the presence of an organic additive-containing supercritical carbon dioxide sufficient to inactivate the microbes and to thereby achieve whole microbe cell walls which are "empty" (i.e., have a lysis hole when viewed by scanning electron microscope (SEM)).

The organic additives employed in the present invention most preferably include alkanecarboxylic acids and/or alkanepercarboxylic

acids, each of which may optionally be substituted at the alpha carbon with one or more electron-withdrawing substituents, such as halogen, oxygen and nitrogen groups. Particularly preferred species of organic additives employed in the practice of the present invention include acetic acid (AcA), peracetic acid (PAA) and trifluoroacetic acid (TFA). One particularly preferred liquid additive that may be employed in the practice of the present invention is commercially available Sporeclenz® sterilant which is a mixture of acetic acid with hydrogen peroxide and peracetic acid.

The organic additive is employed in an effective amount of at least about 0.01 vol.% and greater, based on the total volume of the carbon dioxide. The amount of organic additive will be dependent upon the particular additive that is employed. Thus, for example, peracetic acid may be present in relatively small amounts of about 0.5 vol.% and greater, while acetic acid may need to be employed in amount of about 1.0 vol.% and greater. Thus, a range of at least about 0.01 vol.% and greater, up to about 2.0 vol.% will typically be needed in order to achieve an enhancing effect in combination with carbon dioxide.

One presently preferred embodiment of an apparatus 10 according to the present invention is depicted in accompanying FIGURES 1 and 2. In this regard, it can be seen that the apparatus includes a standard compressed gas cylinder 12 containing carbon dioxide, and a standard air compressor 14 used in operative association with a carbon dioxide booster 16 (e.g., Hasket Booster AGT 7/30). Alternatively, the air compressor 14 and booster 16 can be replaced with a single carbon dioxide compressor.

An additive cycle is also provided by means of a series of an inlet port 18 which allows additive contained in reservoir 20 to be added to a pressure vessel 22 through valve 24 and additive line 26. The carbon dioxide is introduced to the pressure vessel 22 from header line 27 via valve 28 and CO_2 supply line 30. A filter 32 (e.g., a 0.5 micron filter) is provided in the supply line 30 to filter the supplied CO_2. A pressure gauge 34 is provided downstream of CO_2 shut-off valve 36 in supply header 27 to allow the pressure to be visually monitored. A check valve 38 is provided in the line 27 upstream of the valve 36 to prevent reverse fluid flow into the booster 16. In order to prevent an overpressure condition existing in line 27, a pressure relief valve 39 may be provided.

An outlet line 40 allows the pressure vessel 22 to be depressurized. In this regard, the depressurized fluid exits the vessel 22 via line 40, is filtered by filter unit 42 and then is directed to separator 44 where filtered CO_2 gas may be exhausted via line 48, and liquid additive collected via line 50 for possible reuse. Valves 52, 54 may be provided in lines 46 and 27, respectively, to allow fluid isolation of upstream components.

The reactor vessel 22 is most preferably constructed of stainless steel (e.g., 316 gauge stainless steel) and has a total internal volume sufficient to accommodate the materials being sterilized either on a laboratory or commercial scale. For example, in laboratory studies, an internal volume of 600 mL (e.g., approximately 8 inches long by about 2.5 inches in diameter) was deemed adequate As is perhaps more clearly shown in FIGURE 2, the pressure vessel 22 includes a vibrator 60, a temperature control unit 62, and a mechanical stirring system most preferably comprised of an impeller 64 and a magnetic driver 66. The reactor vessel 22 contains a conventional basket (not shown) which is

also preferably constructed of 316 gauge stainless steel. The basket serves to hold the items to be sterilized as well as to protect the impeller 64 and direct the inactivation fluid in a predetermined manner.

The reactor vessel 22 may be operated under constant pressure or continual pressurization and depressurization (pressure cycling) conditions without material losses due to splashing or turbulence, and without contamination of pressure lines via back diffusion. The valves 24, 28 and 52 allow the vessel 22 to be isolated and removed easily from the other components of the apparatus 10. The top 68 of the pressure vessel 22 may be removed when depressurized to allow access to the vessel's interior.

In use, the microbial material to be sterilized is introduced into the interior space of the pressure vessel 22 along with any initial portion of liquid organic additive from reservoir 20. The temperature control unit 62 is operated so as to set the desired initial temperature for sterilization. The vessel 22 may then e pre-equilibrated with carbon dioxide from gas cylinder 12 at atmospheric pressure, following which the magnetic driver 66 is operated so as to activate the impeller 64. The pressure vessel 22 may thereafter be pressurized to a desired pressure by introducing additional carbon dioxide gas from cylinder 12 via the air compressor 14 linked to booster 16.

In order to effect a pressure cycling of the vessel 22, an amount of carbon dioxide may be released therefrom via depressurization line by momentarily opening valve 52 sufficient to partially reduce pressure within the vessel 22. Additive may be introduced into the vessel 22 for any given pressure cycle by opening valve 24 which allows liquid additive to flow from reservoir 20 into inlet port 18. It will be understood that the additives

may be introduced prior to pressurization and/or during pressure cycling. Prior to pressurization, additives are introduced directly into the reactor vessel 22 prior to sealing and/or via the additive port 18. The additives are most preferably introduced during the cycling stages by measured addition to the additive port 18 at ambient pressures. The port 18 is subsequently sealed and the additive chamber is pressurized so that the additive may enter the reactor vessel 22 without altering the internal pressure. The exact mechanism of addition may be modified such that the process is more efficient and/or convenient.

Following additive introduction, the vessel 22 may be repressurized to a desired pressure following introduction of the liquid additive therein. Such depressurization/repressurization with introduction of liquid additive may be repeated for any number of cycles that may be desired.

Most preferably, periodic agitation to the contents of vessel 22 is effected using vibrator 60 through entire depressurization/repressurization cycles. Intermittent or continuous agitation of the reactor vessel and its contents is performed by vibrating the reactor vessel during sterilization. Agitation enhances mass transfer of the carbon dioxide and additives by eliminating voids in the fluid such that the microbial material being inactivated comes into more complete contact with fluid. The specific means of agitation may be adjusted to accommodate the particular apparatus employed and to optimize sterilization times, temperatures, and pressure cycles. When treatment is complete, the vessel 22 is depressurized, the magnetic drive 66 is stopped thereby stopping the stirring impeller 64, and the thus inactivated microbial material removed by opening top 68 of vessel 22.

The present invention will be further understood after careful consideration is given to the following Examples.

Example 1 - Inactivation of bacteria by treatment with supercritical CO2

The process described above and in copending U.S. Provisional Appliation 60/---,--- filed even date herewith (Atty.Dkt. 2805-3) was followed so as inactivate resistant spore forms of *B. subtilis* and *B. stearothermophilus*, with greater than 6 log reductions in under 2 hours.

Example 2 - Inactivation of bacteria by treatment with supercritical CO2

Maintaining the natural presentation environment for a given antigen generally enhances the protective qualities of a given vaccine. Ghost vaccine preparations result in empty bacterial shells that are intact save for a lysis hole when viewed by scanning electron microscopy (SEM)[5, 13, 14]. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were inactivated by treatment with supercritical CO2 at 40^{0}C and 2973-1500 psi for a total of 6 cycles over 4 hours. *Eschericia coli* was inactivated by treatment with supercritical CO2 at 34^{0}C and 2973-1500 psi for a total of 3 cycles over 0.5 hours. As shown by the SEM micrographs of Figures 3A-3F, like the ghost particles, microbes remain intact when inactivated by supercritical CO_2.

Example 3 - Preparation and protein analysis of B. subtilis vaccine

Using the equipment and procedure in Figure 1 and outlined above, 1 mL spore/vegetative preparations of *B. subtilis* of greater than 10^6 CFU/mL was placed into 16mm test tubes in a stainless steel basket. Acetic acid (6 mL) was transferred by syringe onto the surface of a cotton ball placed in basket, and the basket then loaded into the 600 mL reactor

vessel. The reactor vessel was heated to 50^{0}C and equilibrated with CO_2 at atmospheric pressure. The stirring and agitation mechanisms were activated and vessel pressurized to 3000 psi for 40 minutes. Agitation is carried out for 5 minutes. The CO_2 pressure was then allowed to drop to 1500 psi at a rate of 300psi / minute.

Once the vessel is de-pressurized, 4 mL acetic acid is added at ambient pressure to additive loop. The additive loop is sealed and pressurized to 3000 psi. The vessel is then re-pressurized through the additive loop to 3000 psi such that acetic acid is carried into vessel.

The pressurization/stirring/agitation/depressurization/additive addition process was repeated a total of 3 times. After the third cycle, a series of three flushing cycles to remove the additive was performed by pressurizing and de-pressurizing the reactor vessel using CO2. The stirring was stopped and the basket was removed from the reactor vessel. Quantitative analysis of the remaining CFU count vs. untreated control is enumerated though serial dilutions and colony counts.

The impact of the method according to the present invention was evaluated by performing SEM analysis and protein profiling. Specifically, it was observed from Figure 4A that cell walls remain intact. Moreover, extracts of *B. subtilis* spores both untreated and treated with supercritical CO2 are found to be virtually identical. In addition, there appears to be no major depletion of proteins from the bacteria as total protein levels are similar as shown in Figure 4B.

Thus, experiments to date support the likelihood that bacteria inactivated by supercritical CO_2 have potential as high quality whole-cell vaccine preparations. This is supported by the observation that significant log reductions in CFUs are achieved for a wide range of bacteria while the

morphology of bacteria remains intact, proteins are not significantly degraded, bio-degradable polymers are unaffected, and the process is easily scaled up.

****

While the invention has been described in connection with what is presently considered to be the most practical and preferred embodiment, it is to be understood that the invention is not to be limited to the disclosed embodiment, but on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the present invention.

References:

1. Dillow, A.K., et al., Bacterial inactivation by using near- and supercritical carbon dioxide. Proceedings of the National Academy of Sciences of the United States of America, 1999. 96(18): p. 10344-8.

2. Duque, H., et al., Effects of formalin inactivation on bovine herpes virus-1 glycoproteins and antibody response elicited by formalin-inactivated vaccines in rabbits. Vaccine, 1989. 7(6): p. 513-20.

3. Huter, V., et al., Improved protection against lung colonization by Actinobacillus pleuropneumoniae ghosts: characterization of a genetically inactivated vaccine. Journal of Biotechnology, 2000. 83(1-2): p. 161-72.

4. Murphy, B.R. and E.E. Walsh, Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. Journal of Clinical Microbiology, 1988. 26(8): p. 1595-7.

5. Jalava, K., et al., Bacterial ghosts as vaccine candidates for veterinary applications. J Controlled Release, 2002. 85: p. 17-25.

6. Witte, A., et al., Phi X174 protein E-mediated lysis of Escherichia coli. Biochimie, 1990. 72(2-3): p. 191-200.

7. Eko, F.O., et al., Characterization and immunogenicity of Vibrio cholerae ghosts expressing toxin-coregulated pili. Journal of Biotechnology, 2000. 83(1-2): p. 115-23.

8. Eko, F.O., et al., Immunogenicity of Vibrio cholerae ghosts following intraperitoneal immunization of mice. Vaccine, 1994. 12(14): p. 1330-4.

9. Furst-Ladani, S., et al., Bacterial cell envelopes (ghosts) but not S-layers activate human endothelial cells (HUVECs) through sCD14 and LBP mechanism. Vaccine, 1999. 18(5-6): p. 440-8.

10. Haslberger, A.G., et al., Activation, stimulation and uptake of bacterial ghosts in antigen presenting cells. Journal of Biotechnology, 2000. 83(1-2): p. 57-66.

11. Szostak, M.P., et al., Bacterial ghosts: non-living candidate vaccines. Journal of Biotechnology, 1996. 44(1-3): p. 161-70.

12. Eko, F.O., et al., New strategies for combination vaccines based on the extended recombinant bacterial ghost system. Vaccine, 1999. 17(13-14): p. 1643-9.

13. Huter, V., et al., Bacterial ghosts as drug carrier and targeting vehicles. 1999. 61(1-2): p. 51-63.

14. Lubitz, W., et al., Extended recombinant bacterial ghost system. Journal of Biotechnology, 1999. 73(2-3): p. 261-73.

15. Szostak, M.P., et al., Bacterial ghosts as multifunctional vaccine particles. Behring Institute Mitteilungen, 1997(98): p. 191-6.

16. Eko, F.O., et al., Production of Vibrio cholerae ghosts (VCG) by expression of a cloned phage lysis gene: potential for vaccine development. Vaccine, 1994. 12(13): p. 1231-7.

17. Mader, H.J., et al., Endotoxicity does not limit the use of bacterial ghosts as candidate vaccines. Vaccine, 1997. 15(2): p. 195-202.

18. Lubitz, W., Bacterial ghosts as carrier and targeting systems. 2001. 1(5): p. 765-71.

19. Remaut, E., H. Tsao, and W. Fiers, Improved plasmid vectors with a thermoinducible expression and temperature-regulated runaway replication. Gene, 1983. 22(1): p. 103-13.

20. Blasi, U., B. Henrich, and W. Lubitz, Lysis of Escherichia coli by cloned phi X174 gene E depends on its expression. Journal of General Microbiology, 1985. 131 (Pt 5): p. 1107-14.

21. Fitzgerald, J.R., et al., Evolutionary genomics of Staphylococcus aureus: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. Proceedings of the National Academy of Sciences of the United States of America, 2001. 98(15): p. 8821-6.

22. Ge, Y., et al., Extraction of natural vitamin E from wheat germ by supercritical carbon dioxide. Journal of Agricultural and Food Chemistry, 2002. 50(4): p. 685-9.

23. Wu, Q. and W.D. Marshall, Approaches to the remediation of a polychlorinated biphenyl (PCB) contaminated soil--a laboratory study. 2001.3(3): p. 281-7.

24. Fages, J., et al., Use of supercritical CO2 for bone delipidation. Biomaterials, 1994. 15(9): p. 650-6.

25. Subramaniam, B., R.A. Rajewski, and K. Snavely, Pharmaceutical processing with supercritical carbon dioxide. Journal of Pharmaceutical Sciences, 1997. 86(8): p. 885-90.

26. Haas, G.J., et al., Inactivation of Microorganisms by Carbon Dioxide Under Pressure. J. of Food Safety, 1989(2): p. 253-65.

27. Spilimbergo, S., N. Elvassore, and A. Bertucco, Microbial inactivation by high-pressure. J. Supercritical Fluids, 2002. 22: p. 55-63.

28. Fages, J., et al., Viral inactivation of human bone tissue using supercritical fluid extraction. Asaio Journal (American Society For Artificial Internal Organs : 1992), 1998. 44(4): p. 289-93.

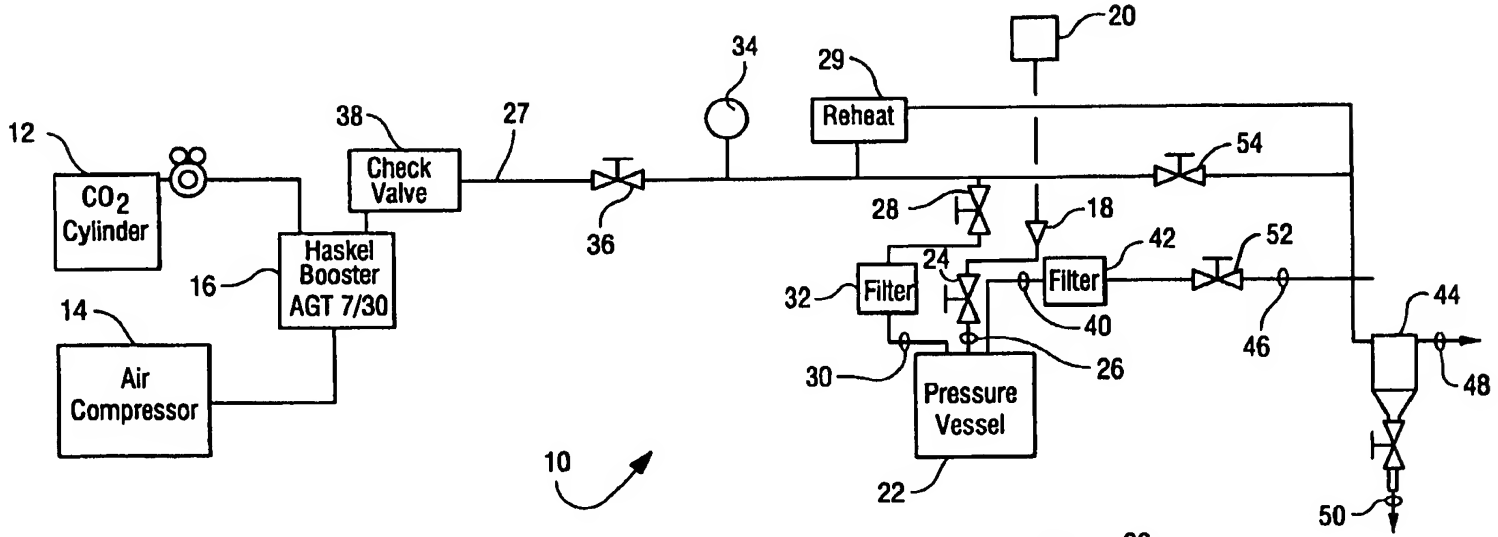

12
CO_2 Cylinder
14
Air Compressor
16
Haskel Booster AGT 7/30
38
Check Valve
27
36
34
29
Reheat
20
54
28
18
42
52
24
32
Filter
Filter
40
46
44
30
26
48
Pressure Vessel
22
50
10

Fig. 1

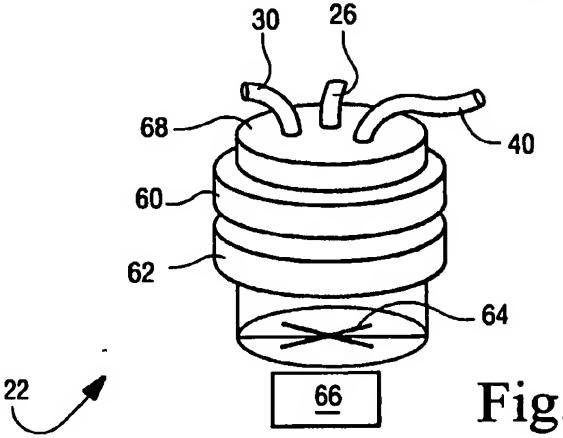

30
26
68
40
60
62
64
66
22

Fig. 2

Fig. 3A

Before

Aft r

Fig. 3B

S. aureus

Fig. 3C

Fig. 3D

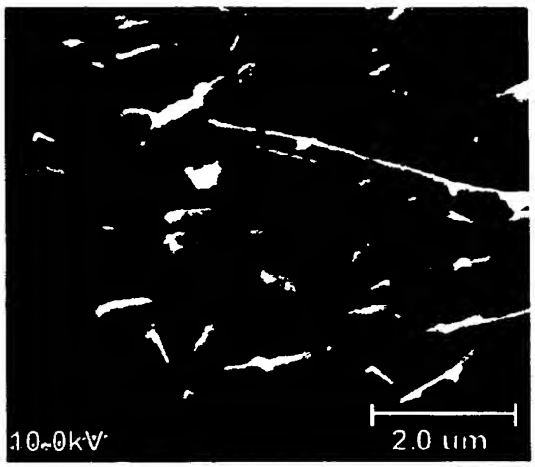
P. aeruginosa

Fig. 3E

Fig. 3F

E. coli

Fig. 4A



B. subtilis

Fig. 4B